Stimulation of blue bilin production by light in the wing of a papilionid butterfly *Graphium sarpedon*

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Abstract Light irradiation stimulates blue coloration in the band region of the adult wing of a butterfly, *Graphium sarpedon* whereas the region remains yellow in darkness. This blue color is due to the presence of a blue bilin pigment (sarpedobilin). In the present study, we show that the amount of blue bilin in the wing band region increases with increasing intensity or duration of light exposure. Further, blue bilin accumulation occurs when local irradiation of light falls on the wing, but not on the adult body. This remained true even when only the wing separated from an adult body or only the limited region of the wing band pattern was exposed to light. Blue light is more effective than green or red light. Microscopic observations show that the blue coloration is derived from part of the wing membrane, but not from wing scales. These results strongly suggest that light acts directly on the wing tissue without the intervention of a neuroendocrine system, and increases the amount of blue bilin in the wing membrane.

Key words *Graphium sarpedon*, butterfly, adult wing, light irradiation, sarpedobilin.

Introduction

Coloration and color polymorphism of insects are often initiated by environmental stimuli such as light, temperature and humidity. In many cases, the environmental signal operates on a neuroendocrine factor, which then regulates the metabolism and accumulation of pigments via gene expression (Raabe, 1983; Hoffmann, 1984; Fuzeau-Braesch, 1985; Kayser, 1985; Riddiford *et al.*, 1990).

Blue/green insects are cryptic on plant leaves against a visual predator, and their color is due to the presence of blue bilin with yellow carotenoid in the integument and hemolymph (Kayser, 1985). Kato and his colleagues demonstrated that green coloration of the cocoons of some saturniid moths, *e.g.*, *Antheraea yamamai* and *Rhodinia fugax* is stimulated by light irradiation during the late larval stage (Kato *et al.*, 1989; Kato and Miyata, 1994). If larvae are kept in darkness, they produce yellow cocoons. It was demonstrated in *A. yamamai* that light irradiation directly stimulates blue bilin production in the hemolymph without the intervention of a neuroendocrine system, following which its blue bilin is transferred into silk glands (Kato, 1991; Yamada and Kato, 2004). This is unlike many other insect species where blue bilin is synthesized without light stimulus (Kayser, 1985).

We also demonstrated that light-induced blue/green coloration occurs in the larval integument and adult wing of a papilionid butterfly *Graphium sarpedon*, which is unrelated to saturniid species (Kato and Yamada, 2001). The larvae of this butterfly are green in color, and may be

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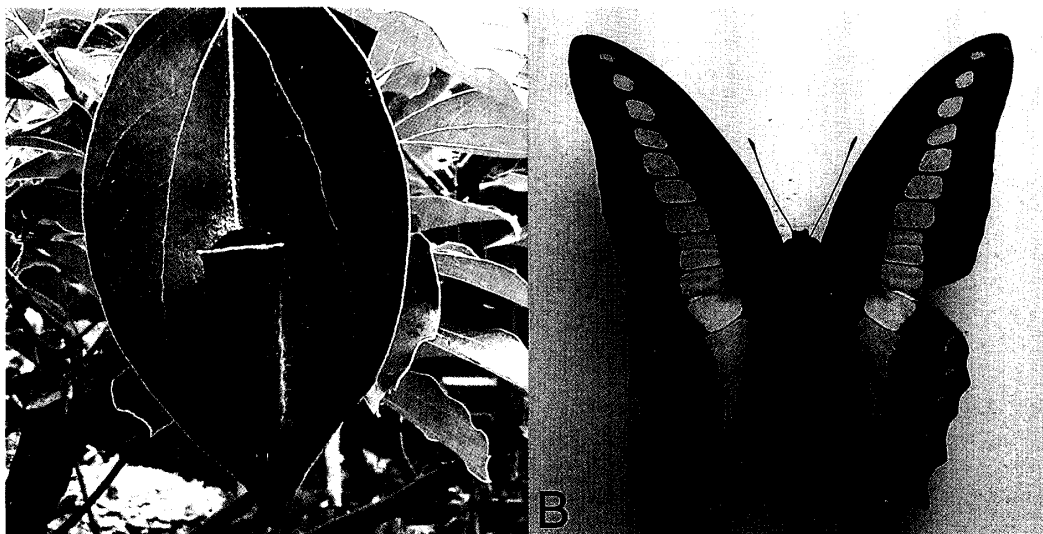†Passed away on February 24, 2010

Fig. 1. *Graphium sarpedon*. (A) A last instar larva resting on a host-plant leaf. (B) An adult specimen (male).

cryptic on host-plant leaves (Fig. 1A). In the adults, the band region of the wing is blue with blackish ground-color (Fig. 1B), and there is no sexual difference in the blue coloration. If enough light is not given just after adult eclosion, the band region remains pale yellow in color. In *G. sarpedon,* the blue color of adult wings plays a crucial role as a visual stimulus in mate searching by the male (Kato and Yoshioka, 2003).

Blue bilin pigment in the wing of *G. sarpedon* (*=Papilio graphium sarpedon*), which is a new kind of pterobilin, was named as sarpedobilin (Choussy and Barbier, 1973; Bois-Choussy and Barbier, 1977). Sarpedobilin pigment was later found not only in the larval integument of the same species, but also in the cocoons of unrelated species, *A. yamamai* and *R. fugax* (Kato and Miyata, 1994; Yamada and Kato, 2004). This pigment is also bound to a specific protein in the wing and other tissues, and its accumulation depends on light stimulation, as described above. Thus, it will be interesting to know why the system of light-induced bilin production might have evolved among these unrelated lepidopteran insects.

The goal of our study is to understand the physiological and biochemical mechanism by which blue/green coloration in these insects is stimulated by light. Here, (1) light conditions responsible for blue bilin abundance in the wing were examined in detail, (2) tests were carried out to ascertain whether the occurrence of this bilin may be induced by the exposure of the wing iself to light or not, and then (3) the adult wing was microscopically observed to investigate the relationship between the blue color and the scale shape.

Materials and methods

Insects

Eggs and young larvae (1st to 3rd) of *G. sarpedon* were collected from host plants (*Cinamomum camphora* and *Machilus tunbergii*) in the fields of Tokyo and neighboring localities. Larvae were reared on leaves of *C. camphora* in transparent plastic cups (11cm in diameter, 5cm in depth) under a photoperiod of 16L-8D at 25°C. During the light phase, light intensity was kept at about 10-20lux. Newly ecdysed pupae were kept in the dark box at 25°C till eclosion. Adults within 24hr after eclosion were designated as day-0.

Light irradiation on intact adults

Live adults of day-1 or later were put singly into transparent plastic cups, and then exposed to light of various intensities during periods of time at 25°C. As the light source, white fluorescent tubes (National, FL SUE/37) were used and the light intensity was measured at the top of the cup with a photometer (Topcon). To obtain the desired intensities of light, we changed the distance from the light source to the rearing cup.

Local irradiation experiments

(1) On the intact adults: Adults of day-1 clipped at the base of the wings were placed horizontally on a white sheet, and only the body part or wings were exposed to the light of 5,000lux for 24hr. The non-irradiated part was covered with thin alminium foil to avoid light exposure.

(2) On the separated forewing: In these experiments, only one forewing per individual was used, unlike the above experiments where two forewings of each individual were analyzed. Forewings of day-1 adults were cut off at the base with fine scissors, and then dorsally exposed to light of 5,000lux for 24hr as above. For local irradiation, a band region or non-band region of the forewings was exposed to light. An entire or half area of one large spot consisting of the band region, which is next to the inner margin of the forewing, was also exposed to light of 5,000lux.

Examination of colored light responsible for blue coloration of a wing

The effect of colored light was examined using the forewings separated from day-1 adults. Each forewing was exposed to blue, green or red light, which was obtained by the use of color filters (Fuji-filter BPB-42 [blue], BPB-50 [green], BPB-60 [red], Fuji-Film Co.), for 24hr at 25°C. Transmission peak was 420nm for the blue filter, 500nm for the green filter and 600nm for the red filter. In this experiment, a mercury lamp (Mitsubishi-Osram, MLRBOC400F-U) was used as a light source to obtain high intensity of light (In this case, light intensity was 10,000lux). Light energy given to a wing, which was measured with a radiometer (United Detector Technology, Model 161), was approximately 7.0×10^{-3} mW/cm^2 for each color.

Determination of the relative abundance of blue bilin in the wing

To evaluate the effect of light irradiation, blue bilin was extracted from the wing, and then its abundance was spectrophotometrically analyzed. All wing samples were kept at –30°C until use.

Two forewings per individual were basically used for pigment analysis, but one forewing alone used for the local irradiation and colored light irradiation experiments where the separated wings were irradiated. Blue bilin was extracted from the whole band region of each forewing or from a single spot area of the band region. These regions were cut into fine pieces, and the pieces were homogenized with a mixture of H_2SO_4 and methanol (1: 9, v/v) in a glass homogenizer for 5 min in an ice bath. This extract was put into a microtube and centrifuged at 10,000 r.p.m. for 5 min at 5°C to remove cuticular and cellular debris. Under these conditions, blue bilin is extracted as an esterified form (presumably, as methyl ester) (Kayser, 1985; Yamada and Kato, 2004). The absorption spectrum was examined with a spectrophotometer (Shimadzu, BioSpec-1600). The amount of blue bilin in each sample was expressed as absorbance value at 662 nm.

Further, we analyzed the blue extract to investigate whether the blue pigment consisted of a single component, using high performance liquid chromatography (HPLC). Ten to 50μl of the extract was injected into a reverse phase column (Asahi-pack ODS-50, Asahi Kasei Co., 6x150 mm, 5μm) and eluted with 0.1% trifluoroacetic acid/methanol (4: 6, v/v) at a flow rate of 1ml

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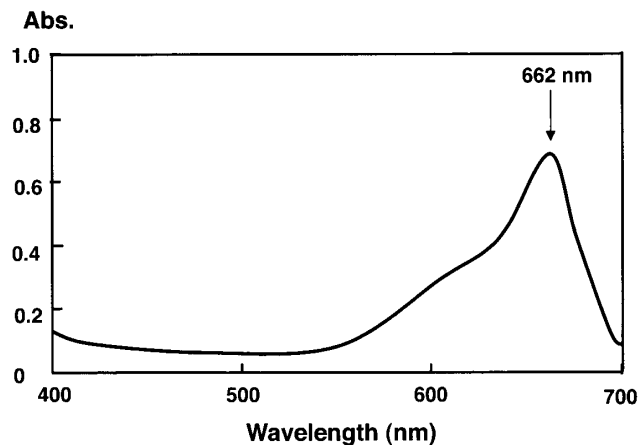

Fig. 2. Absorption spectrum of the blue extract from the wing of a light-irradiated adult. Absorption peak is seen at 662 nm.
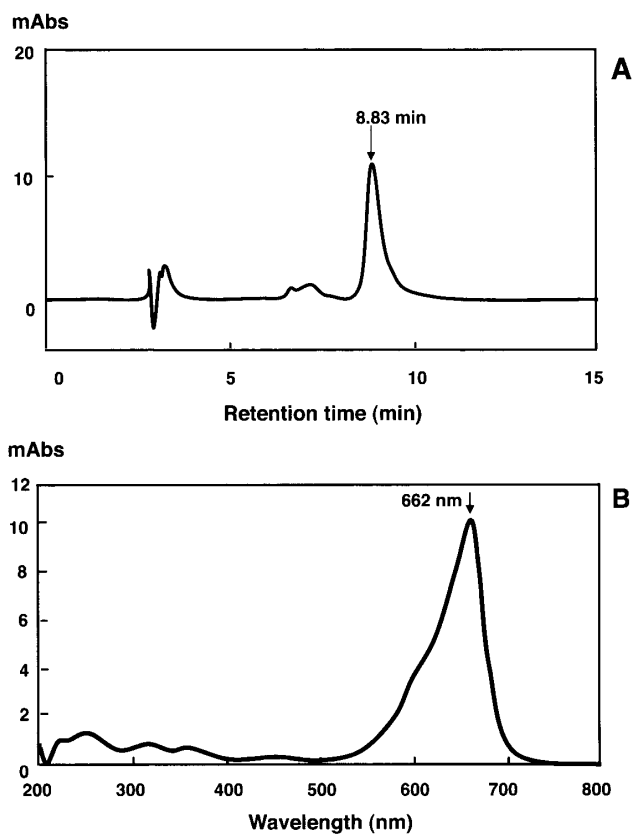

Fig. 3. Chromatogram and absorption spectrum of a bilin ester extracted from the wing of a light-irradiated adult. (A) Chromatogram by HPLC analysis. Absorption was monitored at 670nm. One peak is seen at 8.83min. (B) Absorption spectrum of the peak fraction shown in (A). Absorption peak is seen at 662 nm.

per min at 25°C. The elution component from the column was monitored by a photodiode array (SPD-MA10D) at 670 nm and the spectrum at peak position was recorded.

Microscopic observation of the blue band region of a wing

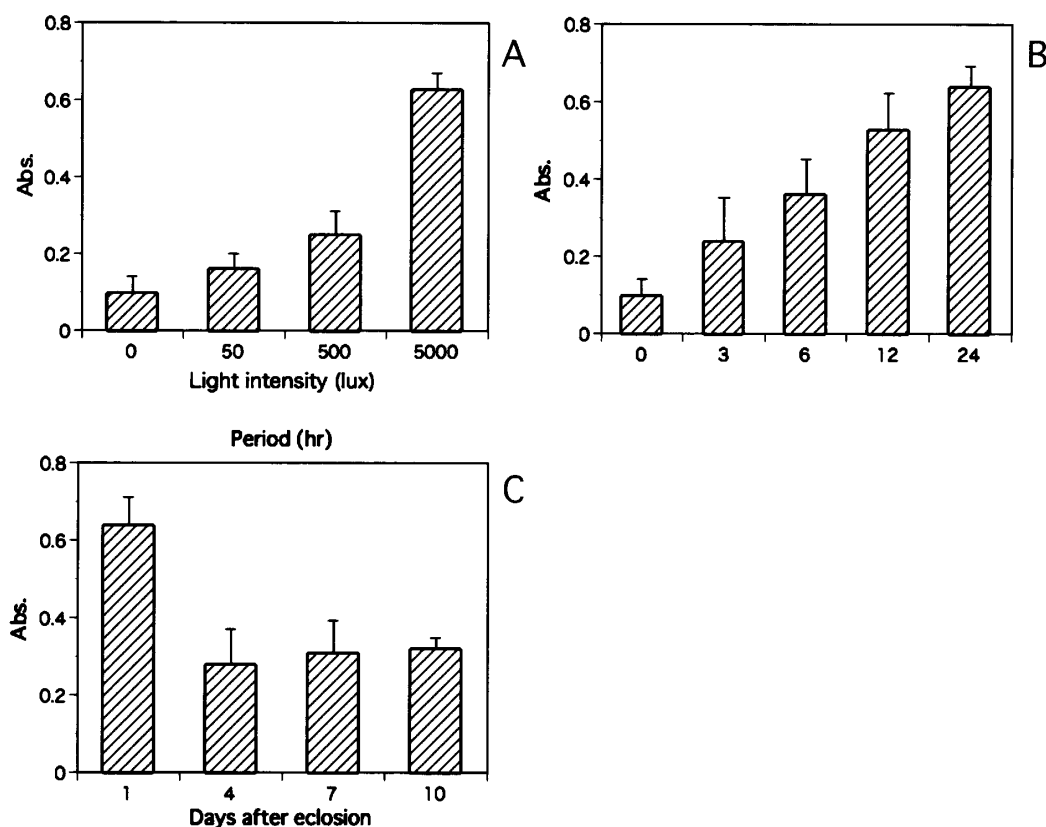To clarify whether or not the blue color might be derived from the scale shaft on a wing, the

Fig. 4. Abundance of a blue bilin ester from a forewing of adults exposed to various light conditions. (A) Exposure to different light intensities for 24 hr. (B) Exposure to light of 5,000 lux for different periods. Numerals above histograms are sample size. (C) Exposure to light of 5,000lux on adults of different ages. Vertical bars show SD. The amount of bilin in the extract is expressed as absorbance at 662 nm. In these experiments, two forewings per individual were analyzed, and total amount was expressed. Sample sizes are 5–7 each. For statistical analysis, (A) $p<0.00001$, (B) $p<0.000001$ and (C) $p<0.005$, Kendall's rank correlation test.

band region of a forewing was cut off from the irradiated or non-irradiated adult body, mounted in glycerol, and then observed under a light microscope (Olympus BX50).

Results

Spectrophotometrical analysis of blue bilin pigment from the wing

Fig. 2 shows the absorption spectrum of the blue extract of the wing before HPLC analysis. This spectrum had one maximum absorbance at 662 nm, and no peak around 400 nm. In Fig. 3A, the HPLC profile of blue bilin extracted with acidic methanol from the adult wing is shown. This blue bilin, which may be the esterified form of the bilin (Yamada and Kato, 2004), was observed as one peak with a retention time of 8.83 min. The absorption spectrum of this peak had a maximum at 662 nm (Fig. 3B). Because the absorption pattern was basically the same in both, we used the former data to determine blue bilin abundance in the wing.

Effect of light irradiation on intact adults

When adults of day-1 were irradiated for 24hr by various intensities of light, blue pigment abundance increased as the intensity was high (Fig. 4A) ($p<0.00001$, Kendall's rank correlation test). In the present experiment, however, the relation between them was not linear. The

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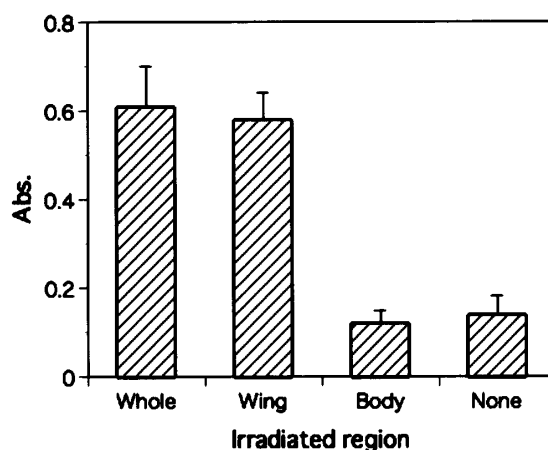
Fig. 5. Abundance of a blue bilin ester from a forewing of adults exposed locally to 5000 lux for 24 hr. Sample sizes are 6–7 each. $p<0.001$ between body and wing irradiations, Mann-Whitney U-test. Other explanations are same as in Fig. 4.

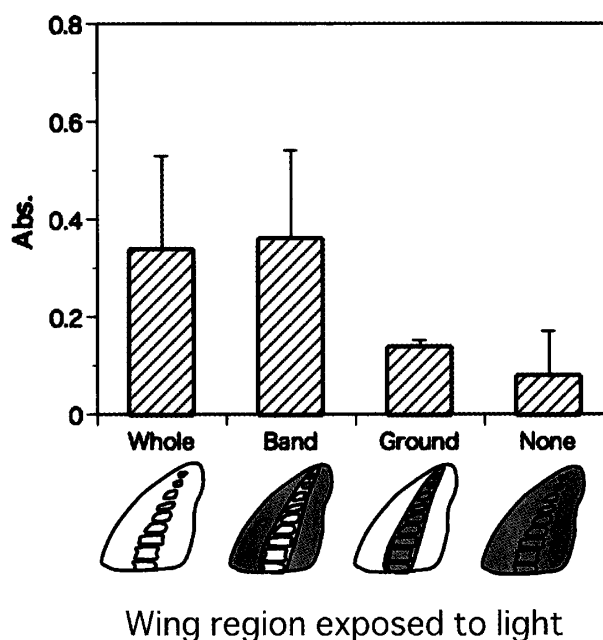

Wing region exposed to light

Fig. 6. Abundance of a blue bilin ester from the forewing isolated from a body part, and then exposed locally to 5,000 lux for 24 hr. In these experiments, one forewing per individual was analyzed. Sample sizes are 4–7 each. $p<0.05$ between whole irradiation and non-irradiation, and between band region and non-band region irradiations, Mann-Whitney U-test. Other explanations are same as in Fig. 4.

reason for this will simply be due to the experimental design. Next, when the adults were exposed to light of 5,000lux for different periods, pigment abundance increased as the period became longer (Fig. 4B) ($p<0.000001$, Kendall's rank correlation test). Third, when adults of various ages (day-1 to day-10) were examined, pigment abundance was high in day-1 adults while it was low in old adults (Fig. 4C) ($p<0.005$, Kendall's rank correlation test). This means that completely dried wings are not responsive to light.
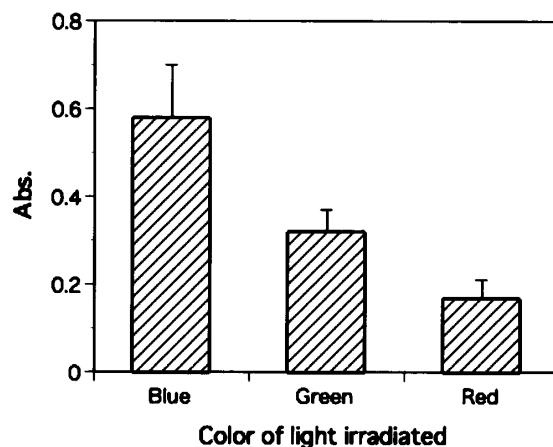
Fig. 7. Effect of colored light on blue bilin abundance in isolated forewings.
Abundance of a blue bilin ester from the forewings exposed to blue, green or red light for 24hr. In these experiments, one forewing per individual was analyzed. Sample sizes are 5 each. A significant difference exists between the three treatments ($p<0.002$, Kruskal-Wallis test). A significant difference is also found between blue and red lights ($p<0.002$, Multiple comparison test). Other explanations are same as in Fig. 4.
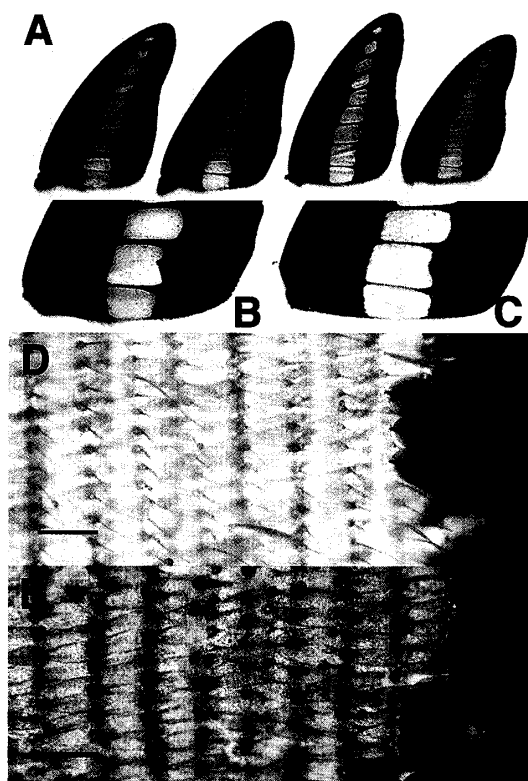

Fig. 8. (A) Forewings of adults exposed to various intensities of light for 24 hr. Blue color becomes deeper as light intensity is higher (from left to right: 5,000 lux, 500 lux, 50lux and darkness). (B) and (C) Local exposure of light on the whole marking (left) and on half of the marking (right) to 5,000 lux for 24 hr, respectively. Only regions exposed to light are more bluish. (D) and (E) Microscopic photographs of the band region on the dorsal surface of forewings of light-irradiated (5,000 lux)(upper) and non-irradiated (below) adults, respectively. Needle-like scales occur in the band region and they are colorless. Blue coloration is mainly restricted to the base of the scales. Normal shaped scales are seen in the black non-band region.
Bars, 50 μm.

Table 1. Relative abundance of blue bilin pigment for local irradiation on one spot area of the forewing.

Experiments	Whole area of one spot		Half area of one spot	
	N	Absorbance (mean, SD)	N	Absorbance (mean, SD)
Exposed	5	0.215, 0.052	4	0.074, 0.004
		*		*
Unexposed	5	0.062, 0.001	4	0.036, 0.001

* $P < 0.05$ for Mann-Whitney U-test

Effect of local irradiation on intact adults

When the body part was exposed to light of 5,000 lux and the wings were unexposed, blue pigment abundance in the wing was very low (Fig. 5). But, in the case where irradiation was carried out on the band region, pigment abundance was significantly high ($p<0.001$, Mann-Whitney U-test) and the wing became blue-colored.

Effect of light irradiation on the forewing separated from a body part

When the isolated forewings were irradiated with light of 5,000 lux, wing blue coloration was induced and pigment abundance was high in contrast with non-irradiated ones (Fig. 6)($p<0.05$, Mann-Whitney U-test). A similar light effect was found in the case of local irradiation on the band region alone, but not on the non-band region ($p<0.05$, Mann-Whitney U-test).

Further, even when the most posterior spot of the band region was exposed to light, blue coloration occurred within the exposed area (Fig. 8B), and blue pigment abundance was clearly high in contrast with the unexposed area (Table 1)($p<0.00$). A light-irradiation effect was also found in the experiment where only half the area of the spot was exposed to light (Fig. 8C; Table 1)($p<0.01$, Mann-Whitney U-test).

Effect of colored light on blue coloration in the separated forewing

In the present experiment, approximately the same amount of light energy per unit area was given for each colored light. Blue light irradiation was most effective for blue pigment abundance while the effect of red light irradiation was very low (Fig. 7). The effect of green light was intermediate between them. The Kruskal-Wallis test showed a significant difference between the three treatments ($p<0.02$). According to the multiple comparison test, a significant difference was found between blue light and red light $(p<0.02)$.

Microscopic observation on the wings of light-irradiated and non-irradiated adults

The results are shown in Fig. 8D and 8E. On the dorsal side of the band region of the forewing, scales were slender and needle-like in shape, and they are colorless and translucent. Scales of usual shape, which are black in color, were seen in the non-band region. In the wings of irradiated adults, the blue area was mainly located at the base of needle-like scales.

Discussion

A previous paper (Kato and Yamada, 2001) demonstrated that light exposure is required for blue coloration of the wing in *G. sarpedon*. In the present experiments, it was clarified that this blue coloration of the wing by light irradiation is due to the production and accumulation of blue bilin pigment in the wing tissue. As the intensity of light increased or as the period of irradiation increased, the amount of the pigment induced increased. This is a novel finding in butterfly wing coloration where wing blue pigment forms after adult eclosion. Similar light-

stimulated accumulation of bilin pigment has been also found in the larval integument and cocoon of some saturniid moths (Kato *et al.*, 1989; Kato and Miyata, 1994; Saito, 2001). Therefore, it is suggested that light-stimulated blue/green coloration associated with the production and accumulation of the blue bilin in lepidopteran insects may be more usual than expected.

Light irradiation on the *Graphium* wing itself also increased the amount of the blue bilin. This increase was found not only under local irradiation of the wings of intact adults, but also on wings isolated from an adult body. Light irradiation on the band region of a wing or on one spot within the band was also effective in increasing the amount of blue bilin. By contrast, light exposure on the adult body alone or on the non-band region of a wing was not effective. We previously showed in the green coloration of *A. yamamai* cocoons that *in vitro* light irradiation on larval haemolymph at a specific developmental stage stimulated the production of cocoon blue pigment (Yamada and Kato, 2004). Further, light irradiation was effective even in temperatures as low as 4°C. Although *in vitro* experiments have not yet been performed on *G. sarpedon*, it is inferred that light acts directly on an unknown precursor substance(s) present in the band-region tissue of the wing, and then stimulates blue bilin production without the intervention of a neuroendocrine system, as in the case of *A. yamamai* (Kato, 1991; Yamada and Kato, 2004).

In the wing of *G. sarpedon*, the blue bilin pigment is bound with a specific protein (Kato and Yamada, 2001) similar other bilin-binding proteins (*e.g.*, Goodman *et al.*, 1985; Kawooya *et al.*, 1985; Haunerland and Bowers, 1986; Hüber et al., 1987; Schmidt and Skerra, 1994; Saito, 1998; Saito *et al.*, 1999; Yamanaka *et al.*, 2000). Although it has also been reported that sarpedobilin is photochemically synthesized from biliverdin pigment liberated from apoprotein in an *in vitro* experiment (Barbier, 1981), whether this reaction occurs in the *in vivo* condition has not yet been proved. We previously showed in the light-induced green coloration of *A. yamamai* cocoons that the precursor substance of blue bilin [presumably sarpedobilin (Yamada and Kato, 1994, 2004)] is bound with apoprotein and this substance is responsive to light (Yamada and Kato, 2004). Thus, the photochemical process of blue bilin production and/or bilin-apoprotein association underlying the light-induced blue coloration of *G. sarpedon* should be investigated in future.

In the present experiments, of the colored lights tested, irradiation with blue light was most effective for the formation of blue coloration and blue pigment . Similar results were obtained for the green coloration of *A. yamamai* cocoons (Kato *et al.*, 1991). The reason for this remains unknown at present. Presumably a specific precursor substance quite sensitive to blue light must be present in the wing tissue.

In many butterfly and moth species, color and wing pattern is generated by the wing scales where specific pigments exist (Nijhout, 1991). In some papilionid and danaid species, however, the wing membrane is colored while wing scales are often colorless (unpublished observations). The present microscopic observations showed that the blue color of the *G. sarpedon* wing, which is produced by light exposure, is derived from the wing membrane, but not from the scale shafts, and that its coloration is mainly at the base of hair-like scales located within the band region of the wing. When the wings are not exposed to light, the wing membrane of the band region remains yellowish or colorless. In the non-banded blackish region of the wing, by contrast, wing scales were flat and colored as with many other butterflies. To date, however, it remains unknown whether the blue pigment is present within the cuticle layer secreted by the socket and/or scale cells, or within the cell bodies themselves.

The present HPLC analysis confirmed that the blue pigment of the wing consists mainly of one component, and that it may be a sarpedobilin-like pigment although its chemical structure

was not shown in the present study. A previous study (Yamada and Kato, 2004) also showed that the blue bilin of the *A. yamamai* cocoon is almost the same as that from the *G. sarpedon* wing. Yamada and Kato (1994) also suggested that the former bilin is quite similar to sarpedobilin, based on NMR data (Yamada and Kato, 1994). As these lepidopteran species are taxonomically unrelated, it is an interesting question whether the same coloration mechanism exists between these species. Further, blue/green coloration in response to light is known to occur in the wing of the Australian papilionid butterfly, *Graphium macleayanus* (Braby, 2000). We have found a similar sarpedobilin-like pigment in the wing of this butterfly while non-responsive *Graphium* species have a blue bilin of a different type (biliverdin-like) (unpublished observation). So it is strongly expected that the presence of the sarpedobilin-like pigment is closely correlated with light-dependent blue coloration of the wing in papilionid butterflies.

The present results also show that the effect of light exposure depended on the age of adult butterflies, and was more effective in adults of day 1 than in those of later stages. At present, the reason for this remains unknown. As butterfly wings gradually became dried after adult eclosion, however, the wing of day-1 adults may have greater amounts of available precursor materials than adults of other ages.

Acknowledgments

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摘　　　要

光照射によるアオスジアゲ翅の青色ビリン色素の生成 (加藤義臣・江場淳子・山田弘生)

1) アオスジアゲハ Graphium sarpedonにおける成虫翅の青色帯は羽化後の光照射により誘起されるが，羽化した成虫が暗所に置かれたままでは，淡い黄色のままである．この青色はビリン色素の一種，サーペドビリンの存在による．本研究においては，さまざまな光照射条件下においてアオスジアゲハ翅の青色着色効果ならびにサーペドビリン量を調べた．

2) 成虫への光照射実験において，翅の青色ビリン色素の量は照射される光の強度や期間が増すと，増加することが明らかとなった．また，照射効果は羽化後1日目の日齢で最も有効であった．

3) 光受容の部位を探るために，成虫への光照射を部分的に行なったところ，翅のみへの光照射が有効であり，胴体への照射は効果がみられなかった．さらに，胴体から切り離した翅全体への光照射のみならず，青色着色予定帯部分への局所的光照射も有効であった．

4) 有効な光波長については，青色光が最も有効であり，次が緑色光であり，赤色光はほとんど効果がなかった．

5) 最後に，顕微鏡観察により翅の着色部位を観察したところ，青色に着色するのは針状の鱗粉本体ではなく，翅の膜部分 (特に，鱗粉の付根部分) であることが判明した．

6) これらのことから，光は感覚/神経内分泌系を介して翅の青色化に作用するのではなく，翅組織に直接に作用して青色ビリン色素の合成を促すことが示唆される．